Thesis for the degree of Licentiate of Engineering

In vitro characterization of nanodrugs at model lipid membranes

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Abstract

The use of nano-sized drug carriers to improve the efficiency of drug delivery has become well established during the past decades. New nanoparticle (NP) formulations for the administration of biopharmaceuticals (*e.g.* proteins and peptides) emerge at an increasing rate and the need for methods to evaluate their properties is expanding. Rational design of drug carriers requires understanding of their biophysical interactions with various biological barriers, *e.g.* cell membranes, mucus layers, or the blood brain barrier, since most carriers aim to deliver drugs across one or more of such barriers. The shape of NPs and the way they adhere to the cell membrane are important determinants for triggering of endocytosis. Another important NP parameter is their responsiveness to changes in the ambient environment when entering intracellular compartments *e.g.* the endosome or the cytosol.

In this thesis, an *in vitro* screening platform for studying of NP – lipid membrane interaction is presented and used to characterize insulin-loaded polymeric NPs with respect to their interaction with differently charged supported lipid bilayers. By combining different surface sensitive techniques (quartz crystal microbalance with dissipation monitoring, reflectometry, and atomic force microscopy), structural properties of nano-sized polyelectrolyte complexes upon adsorption to model membranes were studied.

From the results it is clear that electrostatic forces are important for the outcome of the NP-lipid membrane adsorption process. Polyelectrolyte complexes, which are non covalent assemblies of oppositely charged polyions, adopted different shapes on different membranes. Upon strong electrostatic attraction between the NPs and the membrane, NPs collapsed into a thin layer on top of an oppositely charged model membrane. This rearrangement process is potentially unfavorable for uptake into epithelial cells through endocytosis. NPs based on polymers with disulfide linkages in the polymer backbone were responsive to reducing agents. This property was shown by exposing membrane-adsorbed bioreducible poly(amido amine) based polyelectrolyte complexes to glutathione, mimicking an intracellular reductive environment. Similarly, the responsiveness of the NPs towards a decrease in ambient pH, mimicking the low pH in the late endosome, was shown.

These results show the application of an experimental platform based on engineered supported lipid membranes and surface sensitive analytical techniques to evaluate drug carriers with respect to their membrane interactions as well as their responsiveness. The information gained from screening of novel drug carries gives important guidance during the process of design and development. An important next step in the development of the presented platform will be to establish a correlation to *in vitro* cell culture assays. NPs for other purposes could also be evaluated.

Keywords: nanomedicine, drug delivery, nanoparticle, drug carrier, supported lipid bilayer, cell membrane, QCM-D, in vitro screening

List of abbreviations

AFM	atomic force microscopy
DLS	dynamic light scattering
EPR	enhanced permeability and retention
LB	Langmuir-Blodgett
LS	Langmuir-Schaeffer
NP	nanoparticle
PEC	polyelectrolyte complex
PEG	polyethylene glycol
POEPC	1-palmitoyl-2-oleyl-*sn*-glycero-3-ethylphosphocholine
POPC	1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphocholine
POPS	1-palmitoyl-2-oleyl-*sn*-glycero-3-phospho-L-serine
QCM-D	quartz crystal microbalance with dissipation monitoring
RES	reticuloendothelial system
SEM	scanning electron microscopy
SLB	supported lipid bilayer

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Appended papers

Paper I

Structural rearrangements of polymeric insulin-loaded nanoparticles interacting with surface-supported model lipid membranes

Rickard Frost, Christian Grandfils, Bernardino Cerda, Bengt Kasemo, and Sofia Svedhem

Submitted to Colloids and Surfaces B: Biointerfaces

My contribution: I planned and performed all of the experimental work apart from the SEM analyses. I wrote the main part of the manuscript.

Paper II

Bioreducible insulin-loaded nanoparticles and their interaction with model lipid membranes

Rickard Frost, Gregory Coué, Johan Engbersen, Michael Zäch, Bengt Kasemo, and Sofia Svedhem

Submitted to Journal of Controlled Release

My contribution: I planned and performed all of the experimental work apart from the polymer synthesis and drug loading efficiency measurements. I wrote the main part of the manuscript.

Paper III

Monitoring of surface interactions as a tool for nanoparticle design

Rickard Frost, Christian Grandfils, Bengt Kasemo, and Sofia Svedhem

Paper contribution for the 11th European Symposium on Controlled Drug Delivery, accepted for publication in J. of Controlled Release

My contribution: I planned and performed all of the experimental work. I took part in the writing process.

1. Introduction

Nano-sized drug carriers are predicted to greatly improve drug administration during the 21st century.[1] To develop new, more efficient and less toxic drugs is an extremely costly and time consuming process and therefore, in most cases, not considered as an option. Instead, existing drugs are often reformulated to improve their pharmacokinetics, *i.e.* the fate of the drug after administration. One way to achieve this is to use a carrier that allow drugs to more efficiently be delivered across biological barriers, and even targeted to the diseased tissue.[2] This rapidly developing methodology holds a great promise because of its potential to affect (i) the biodegradation, (ii) the bioavailability and (iii) the potential side effects of a drug in a positive way.

Rapid biodegradation of biopharmaceuticals in the bloodstream is a common problem that prevents a long lasting therapeutic effect, often asking for a protection of the active substance.[3] The bioavailability of the drug, *i.e.* the fraction of the delivered dose that reaches the circulation system, depends on the specific properties of the drug and the chosen route of administration. There are several different routes to administer drugs, *e.g.* orally, pulmonary, or by injections. Some are more convenient than others and because of this they are more often preferred. By the use of drug carriers it could potentially be possible to alter the route of administration when developing new formulations, *e.g.* the administration of insulin by pulmonary delivery instead of by subcutaneous injections.[4] It is also preferred to administer the smallest possible dose to obtain the desired effect since the risk of adverse side effects (toxicity) is decreased. By targeted drug delivery, a local high dose of the drug at its intended site of action can be reached while healthy tissues in other areas of the body are exposed at lower concentrations. This lowers the risk of adverse side effects tremendously. These drug carriers can, for example, also be designed as contrast agents for different imaging techniques.[5] An additional advantage of small doses is that drug molecules in general and biopharmaceuticals in particular, are expensive to prepare. If only a small amount of the drug were needed the cost could be kept low.

Different materials are used to fabricate the drug carriers and depending of the properties of the drug it could be incorporated in the carrier in different ways. Apart from the chemical properties of the carrier, physical properties like size and shape are also important determinants for its biological functions.[6] Often the size of the materials used is in the nanometer length scale and hence they are referred to as nano carriers or nanoparticles (NPs), where the latter term could include both the carrier and its drug load.

1.1. Understanding of biological barriers

As mentioned above, NPs often need to overcome different biological barriers, *e.g.* mucus layers or cell membranes, to reach their intended target. These tasks are not trivial to achieve and understand, since the barriers are very effective and complex systems. However, it has been shown that nano-sized material can translocate across cell membranes and reach the cytoplasm or even the cell nucleus.[7]

To better understand the fundamental aspects of the NP - cell membrane interaction process the degree of complexity must be held at a minimum. One common methodology is to use a model system of the native cell membrane (see section 3.2).[8-10] The basic feature of the cell membrane is a lipid bilayer, a structure that could be formed on hydrophilic supports such as SiO_2.[11] A supported lipid bilayer (SLB) is one commonly used model system for the cell membrane which could be studied by a variety of different surface sensitive analytical techniques, *e.g.* QCM-D and AFM.[12,13] This type of model system has been used in this work and the interactions with various polymeric drug carriers have been evaluated. Apart from studying the NP-SLB interactions, the engineered responsiveness of NPs towards changes in the ambient environment, mimicking different intracellular conditions, have been evaluated.

In this work, three different types of phospholipids have been used to form model membranes; these are 1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleyl-*sn*-glycero-3-phospho-L-serine (POPS) and 1-palmitoyl-2-oleyl-*sn*-glycero-3-ethylphosphocholine (POEPC) (Figure 1). POPC and POPS occurs naturally in cell membranes, while POEPC is produced synthetically. POPC is a zwitterionic lipid with a net neutral charge. POPS and POEPC hold a net negative and positive charge respectively. Synthetic cationic lipids, *e.g.* POEPC, with various hydrophobic and hydrophilic regions have been developed for liposomal gene delivery. In lipoplexes, complexes between lipid structures and nucleic acids (*e.g.* DNA), electrostatic interactions play an important part.[14] Because of this cationic lipids are needed to attract the polyanionic nucleic acids.

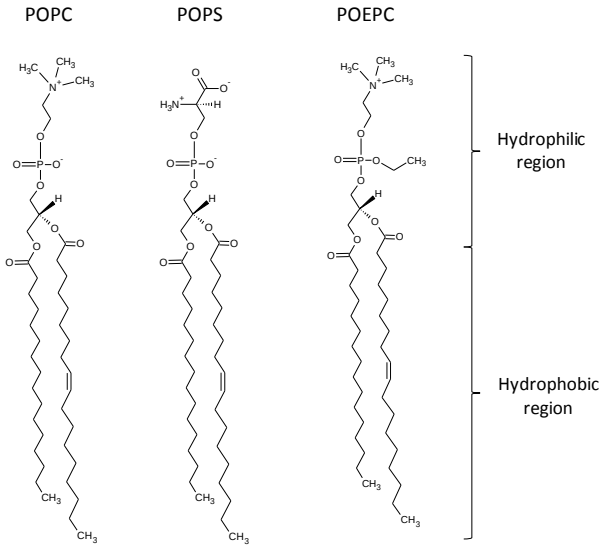

Figure 1. Chemical structures of the three different lipid molecules used in this work.

By selecting these three types of phospholipids it is possible to produce SLBs with different charge. Here, liposomes of POPC:POPS (3:1), POPC:POEPC (3:1) and plain POPC have been used to form SLBs. The complexity of the resulting SLBs is of course low compared to native cell membranes witch to a large extent also contain other components, see section 3.2.2 for how to further increase the complexity. Depending on the properties of the studied NPs, interactions with one or more of the studied SLBs could be expected. Despite this, the

possibility to tune the charge of the model membrane makes it possible to evaluate the importance of the electrostatic attraction for membrane interactions. Furthermore, the integrity of the NPs upon adsorption and possible drug release could be studied as a function of membrane charge.

The main focus in the presented work has been on polymeric NPs loaded with human insulin. Some of these NPs were not functionalized in a specific way while others contained PEG-chains to shield the particles from surrounding substances or were designed to disintegrate when exposed to a reductive agent. According to the description in the next chapter these particles belong to the first and second generation of drug carriers depending on their functionalities.

1.2. Aim

The aim of the work presented in this thesis, and of still ongoing research, is to establish an in vitro screening platform of nanomaterials to study their interactions in model systems involving supported lipid bilayers. This experimental platform consists of a nanomaterial to be studied, a model membrane confined to a solid support and surface sensitive analytical techniques. This platform is expected to yield novel input for the design of new NPs for drug delivery with respect to their intrinsic properties and the way they interact with various biological barriers, both during the process of nanodrug development and production, and as a screening tool to identify potentially toxic nanomaterials.

2. Nanoparticle-based drug delivery

The merging of nanotechnology and medicine is commonly referred to as Nanomedicine[15]. It is a rapidly expanding field of research including many different applications, such as drug delivery systems[16], contrast agents for *in vivo* imaging[5], sensor platforms for *in vitro* diagnostics[17], as well as medical implants and scaffolds for tissue engineering[18]. There is a lot of hope and promise put into the field of Nanomedicine, but it is important to distinguish between the objectives that could be realized within a reasonable timescale, for the benefit of common health, and plain fiction. The great potential of this area arises from the fact that the length scale of the nanomaterials coincides with the length scale of the smallest functional entity in the cell, the proteins. The engineering of functional materials in the nano scale is predicted not only to provide a more effective interaction with living cells but also to reach intracellular targets.

Many of the applications within Nanomedicine are based on the use of nano-sized particles.[19] For example, NPs are designed as drug carriers to protect, target, and release an active substance at the target site, thus minimizing adverse systemic effects.[20] How is this achieved? Obviously, the NP is loaded with the drug that is going to be delivered. This construct, which consists of only a nano sized carrier and a drug, is referred to as the 1st generation of NPs for drug delivery (Figure 2A).

Apart from the drug load, the NP could also be functionalized with a targeting entity (Figure 2B and C). This entity could for example be an antibody, part of an antibody, a peptide, or an aptamer. The targeting molecules then direct the drug to a specific organ or a cell type which express the ligand to the targeting molecule on the cell surface. For example, tumor biomarkers such as epidermal growth factor (EGF) receptors could be targeted for cancer treatments.[21]

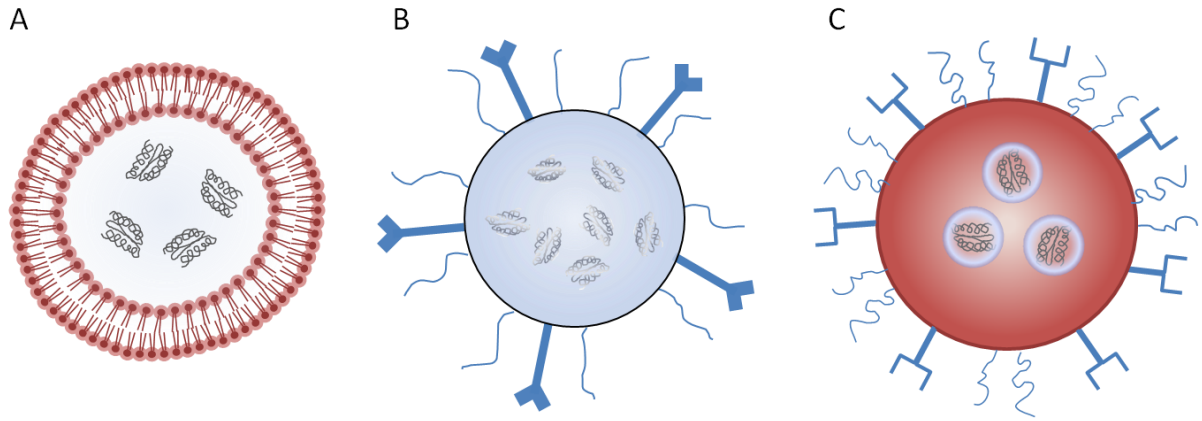

Figure 2. The 1st, 2nd and 3rd generation of drug carriers. (A) The 1st generation consists of a carrier and its drug load. (B) In the second generation the drug carriers are functionalized, *e.g.* with shielding and/or targeting entities. (C) The third generation drug carriers contain several payloads with different targets.

When solid tumors are targeted it is possible to reach a high local concentration of NPs in the tumor, due to the enhanced permeability and retention (EPR) effect, without a specific targeting moiety. The EPR effect relates to the leakiness of the vascular tumor tissue.[22] The targeting of the drug to the diseased tissue rather than having an even distribution it in the entire body increases the efficacy of the drug and reduces the risk of adverse side effects. At the site of action, the release of the active substance could either be passive or initiated by different methodologies depending on the nature of the NP. An active drug release could be achieved by heating absorbing particles through irradiation with ultrasound or light[23] (which could also be the actual therapy) or by enzymatic cleavage[24]. In the latter case the carrier must be engineered so that it dissociates or releases the drug in any other way as a response to a specific enzyme present at the target site. A third approach to an active drug release is to use the local environment in tumors as a trigger, since tumor tissue normally has a lower pH compared to healthy tissue.[25]

When the particle is functionalized with a targeting molecule and/or an active release mechanism they are referred to as the 2nd generation of NPs for drug delivery (Figure 2B). The NPs could also be functionalized in additional ways. For example, it has been shown that pegylation of NPs can significantly prolong their residence time within the bloodstream (a necessary step in the direction towards so called stealth drugs).[3,26] In this way, the foreign material is protected from opsonization and further elimination/degradation by the reticuloendothelial system (RES). When toxic substances are to be delivered, the body should be protected from the substance in a similar manner.

Even more advanced approaches aim at multifunctional particles combining therapy and diagnostics (theranostics), such as magnetic particles which can be used both as contrast agents in MR imaging, and local treatment by the application of an external magnetic field. NPs which belong to the 3rd and, so far, most advanced generation of NPs for drug delivery have the ability to overcome multiple biological barriers (Figure 2C). For example, particles could have multiple payloads where the first payload which is released at the target site is a NP which in turn carries the active substance further in to the diseased tissue.

2.1. Common classes of nanoparticles for drug delivery

Typical NP drug carrier systems include polymeric materials, inorganic materials and liposomes.[20] Size, shape and surface chemistry of the NPs are important components to design their biological properties. The main challenge when designing a carrier for biopharmaceuticals is to formulate the drug load in a proper way. The drug has to be stable when it is attached to the carrier and active when it is released. In the following a few common classes on NPs are reviewed.

2.1.1. Liposomes

In a liposome both hydrophilic and hydrophobic environments are present which enable loading of drugs with totally different properties. Hydrophilic drugs could be loaded in the closed compartment inside the liposome and hydrophobic drugs in the interior of the lipid bilayer. Liposomes are the most common nanocarrier formulations used in clinic today.

Liposomal Doxorubicin, which is used to treat Kaposi´s sarcoma, metastatic breast cancer, advanced ovarian cancer, and multiple myeloma, was among the first NP formulations on the market. The advantage of delivering the Doxorubicin in liposomes instead of as a pure drug is evident when looking at the circulation half life. Free Doxorubicin has a half life of 0.2 h in the circulation system but this time increases to 2.5 and 55 h if the drug is administered in an unpegylated or a pegylated liposome respectively.[27] Furthermore, by using liposomes as drug carriers an active drug release can be achieved at inflamed or cancerous tissue due to the enhanced activity of the secretory phosphlipase A2 (PLA2).[24]

2.1.2. Polymeric nanoparticles

There are many types of NPs based on organic polymers.[28] Polymeric micelles, polymeric vesicles and dendrimers are some of the main groups. Another commonly used type of polymeric NP is polyelectrolyte complexes (PECs) which are created by mixing oppositely charged polyions, where one is the biopharmaceutical to be administered. PEC particles form spontaneously when the components are mixed and are mainly held together by strong electrostatic interactions. However, other interaction forces like hydrogen bonding, hydrophobic interactions and van der Waals forces complement the electrostatic interactions in PEC formation.[29] Physical properties like hydrodynamic diameter, zeta potential, and polydispersity of the PECs are dependent on concentration, ionic strength, pH, and properties of the used polymers. Major advantages of using PECs for drug delivery purposes are that they have a slow rate of degradation, are prepared in water solutions, and do not alter normal cell function.[29] Polycationic polymers have been widely explored for the use in PEC drug delivery systems since DNA, RNA, and most proteins are negatively charged at physiological conditions. Polymers commonly used in such drug delivery systems include biopolymers *e.g.* alginate[30] and chitosan[30-33] but also synthetic polymers *e.g.* poly(dimethylaminoethyl methacrylate)[34] and poly(amido amine)s (PAAs)[35]. In this work several different PECs have been studied. These particles have been developed for non invasive, *e.g.* oral, pulmonary, or nasal administration of human insulin.

2.1.3. Magnetic nanoparticles

Magnetic NPs can be used for many different medical applications. Due to their magnetic properties they can be used as contrast agents for magnetic resonance imaging (MRI) or for magnetic separation purposes, but also to induce hyperthermia or as guidable drug carriers. The particles are most often made of iron oxide, magnetite (Fe_3O_4) or maghemite (γ-Fe_2O_3) and show magnetic properties only when exposed to a magnetic field. When used as drug carriers, magnetic NPs can be concentrated at the target site by a magnetic field. Either a permanent magnet is implanted into the tissue or an external magnetic field is applied. For the magnetic NPs to be attracted by a magnetic field it is important that they are big enough. Too small particles (10-15 nm) are hard to attract towards the blood flow. Other parameters that determine the movement of the NPs are the magnetic field strength and its gradient. Apart from being guided to the diseased tissue, the magnetic NPs can be visualized (diagnostics) and heated by an alternating magnetic field to induce hyperthermia or promote drug release (therapy). [36,37]

2.2. Delivery of insulin using nanoparticle formulations

Diabetes mellitus is a family of diseases in which the blood glucose levels are too high. The two main causes of diabetes mellitus are (1) a deficient production of insulin due to an autoimmune destruction of insulin producing β-cells in the pancreas and (2) an insufficient production of insulin to reach the desired effect, often caused by lack of response to the secreted hormone. These two causes are referred to as type I and type II respectively. Diabetes mellitus is a chronic condition which affects a tremendous number of people worldwide every year. Since insulin was first extracted from the pancreas in 1921 by Banting and Best, huge efforts have been made to administer the hormone in a convenient way for the treatment of the disease. The most common way to administer insulin is through subcutaneous injections and despite the inconvenience for the patients, no alternative routes of administrations have been successful so far. As early as in 1924 the first studies on inhaled insulin were published[44] and since then pulmonary and other non-parental routes of administration, such as transdermal, nasal, oral, and rectal have been investigated.[4,45] In January 2006, Exubera®, an advanced method by Pfizer/Nektar Therapeutics based on recombinant insulin for inhalation, was approved by both the European and the American drug agencies (EMEA and FDA). Although it was the first product of its kind on the market it was withdrawn in October 2007 due to low market acceptance.[46] Other devices for inhaled insulin has reached phase III in clinical trials. One such product was AERx® iDMS developed by Aradigm Corporation and Novo Nordisk. However, Novo Nordisk stopped all investigations on inhaled insulin shortly after Pfizer announced that Exubera® was being withdrawn from the market.

Insulin is often used as a model protein to develop NP formulations for proteins and peptides with the aim to reach a high bioavilability of the drug using non-parental routes of administration. Large focus lays on oral delivery due to its ease of administration and patient compliance. Materials that have been used to formulate insulin for oral delivery include polymeric hydrogels[47], polymeric solid nanoparticles[48] and liposomes[49]. PECs have also been prepared for non-invasive insulin delivery.[30,50,51] In this work several different insulin loaded PECs have been studied with respect to their lipid membrane interactions.

2.1. Endocytosis of nanoparticles

Apart from targeting the drug carrying NP to the diseased tissue, it is often required that the NP and/or its drug load enters the cell to reach a therapeutic effect. The dominating process in which particles are taken up by a cell is called endocytosis.[38] This process of cellular entry can be divided in three main parts. First, the NP is engulfed by the cell membrane forming membrane invaginations which in turn are released into the interior of the cell forming an endosome. Second, the endosomes are delivered to intracellular structures which enables sorting of the NPs to their final destinations. Last, the NPs are delivered either to various intracellular compartments, back to the extracellular environment or through the cell (transcytosis). Another way that NPs can pass the epithelial lining, from the apical to the basolateral side, is in between neighboring cells (paracellular transport, Figure 3A). One physical property of NPs that is an important determinant fate of the endosome is the charge. It has been shown that cationic biodegradable polymeric NPs passed through MDCK

epithelial cells (transcytosis) while anionic particles ended up in lysosomes. The route of entry into the cells was also dependant on the cell type.[39,40]

Endosytosis is divided into two subsections called phagocytosis and pinocytosis (Figure 3B). While phagocytosis mainly occurs in specialized phagocytes, *e.g.* macrophages and neutrophils, pinosytosis occurs in all cell types. Different subtypes of pinocytosis are often classified according to the protein involved in the process. This classification gives rise to clathrin dependent and clathrin independent endocytosis. Furthermore, the clathrin independent pathway is divided into caveolae-mediated endocytosis, caveolae and clathrin independent endocytosis and macropinocytosis.

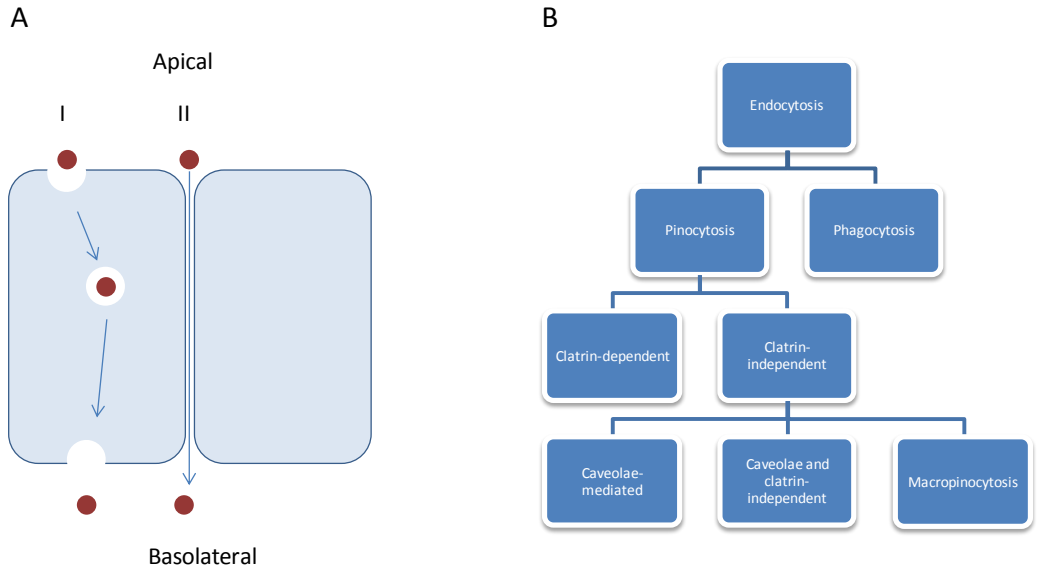

Figure 3. (A) Schematic figure showing the concept of (I) transcytosis and (II) paracellular transport of NPs. (B) Classification of endocytosis.

The two endocytotic pathways which are often considered for uptake of NPs are the clathrin dependent and the Caveolae mediated. Clathrin dependent endocytosis is present in all mammalian cells and responsible for nutrient uptake, *e.g.* iron via the transferrin receptor and cholesterol via the low density lipoprotein receptor. Such natural processes can be utilized for targeting NPs towards endocytosis by decorating them with the appropriate ligands (*e.g.* transferring) although it cannot be known that the fate of the endosome will be the same if targeted NPs are encapsulated.[38]

Caveolae-mediated endocytosis occurs amongst others in endothelial cells and fibroblasts. Caveolae are a special type of lipid raft rich in cholesterol, sphingolipids and proteins like caveolin-1, cavin and dynamin which all are involved in the endocytosis process. The main feature of caveolae-mediated endocytosis that is of importance to nanomedicine is that it can bypass lysosomes and is prominent for transcytosis. Pathogens such as bacteria and viruses utilize caveolae-mediated endocytosis to avoid degradation.[41] Hence, the same process is believed to be beneficial for delivery of biopharmaceuticals.[42] One possible target for nanodrugs that has been identified in the caveolae is aminopeptidase P. [43]

3. Cell membrane mimics

This section briefly describes the native cell membrane with respect to composition and functionalities. The complexity of these systems motivates the use of membrane model systems to learn more about processes involving the cell membrane. Three of the most common model systems liposomes, supported lipid bilayers (SLBs), and Langmuir Blodgett films are described.

3.1. The cell membrane

The cell membrane is a fluid, semi permeable barrier surrounding the cell and separating the intracellular space from the extracellular environment. It is based on amphiphilic lipid molecules, *i.e.* molecules with one hydrophobic and one hydrophilic region. In a lipid membrane the lipid molecules are arranged in a two–layered shell (a bilayer) where the hydrophobic parts are facing each other in the interior of the membrane whereas the hydrophilic parts are exposed to the surroundings. The same structure is also found in many intracellular compartments (organelles) where it serves to separate these compartments from the cytoplasm, *e.g.* the cell nucleus, the mitochondria, the golgi apparatus, the endoplasmatic reticulum, the endosome, and the lysosome.

Although the structure of the cell membrane is based on phospholipid molecules it also contains other molecules like proteins, glycoproteins, cholesterol and glycolipids. The amount of protein associated with a membrane differs between different cell types and organelles, although the typical protein content is about 50 % by mass.[52] The lipid membrane is often described by the fluid mosaic model which was introduced in the early 1970´s where the lipid molecules and the associated proteins are allowed to diffuse freely within the membrane.[53] Although the fluid mosaic model still holds in many respects, the complexity of the cell membrane is today believed to be much greater. The cell membrane contains a large number of different lipid molecules that in some areas are heterogeneously distributed within the membrane, *i.e.* the cell membrane contains domains with different lipid compositions. These domains (sometimes referred to as rafts) could possibly be functional since their properties, *e.g.* with respect to lipid packing, are altered compared to the surrounding membrane.[54]

Proteins associated to the cell membrane, integral and peripheral, allow the cell membrane to carry out a wide variety of different functions. These include transporting nutrients into the cell and waste products out, pump ions or molecules against concentration gradients to keep a proper pH and osmotic pressure inside the cell, form strong connections between neighboring cells to strengthen tissues and create anchoring points for the cytoskeleton to strengthen the cells. Another very important task is carried out by the membrane bound receptors which mediate signals from the surroundings, by binding signaling molecules (hormones or growth factors), to the cytosol leading to various cellular responses. As the membrane proteins play a key role in the cell by sensing the external environment they are important drug targets and much effort is put into studying this class of proteins. About 50% of the drugs on the market

target membrane proteins.[55] Due to the complexity of the native cell membrane, model systems are commonly used to study its properties.

3.2. Membrane model systems

There is a strong need of better understanding the processes taking place at the cell surface. How does a specific protein interact with its membrane bound receptor? What mechanisms determine the way a foreign nano-sized particle interacts with a cell membrane? Questions like these are hard to assess when working with living cells due to the complexity of the system. By using a model system, the degree of complexity could be decreased tremendously and the interaction processes could be studied by a wide variety of analytical techniques, which are not applicable to entire cells. Following this methodology a more fundamental understanding of the interaction processes is gained. Common membrane model systems include liposomes, supported lipid bilayers, and Langmuir-Blodgett films.

3.2.1. Liposomes

Liposomes are spherical entities that consist of a lipid bilayer shell enclosing an aqueous interior.[56] It is possible to produce liposomes in a wide range of sizes, from diameters of a few tenths of nanometers to several hundred micrometers. Often liposomes are referred to as lipid vesicles and classified according to their size and lamellarity. This classification yields small (SUV), large (LUV) and giant (GUV) unilamellar vesicles. Although several different techniques can be used to produce unilamellar vesicles, extrusion is the most common. In this technique, which was first introduced by Hope *et. al.* in 1985[57], a lipid suspension is pressed back and forth through a polycarbonate membrane with a well-defined pore size. Later, in 1991, a convenient device for extruding liposomes in volumes up to 1 mL was described and evaluated.[58] By changing the pore size of the membrane the final size of the liposomes can be tuned. The main advantage of using liposomes instead of supported lipid bilayers is that an aqueous environment is present on both sides of the lipid bilayer. Due to this it is possible to incorporate large transmembrane proteins in their native conformation into the lipid bilayer shell of the liposomes. However, it is difficult to probe the interior compartment of the liposome which would be necessary for measuring for example charge translocations across the membrane.

3.2.2. Supported lipid bilayers

In this work, SLBs were used as a model system for the native cell membrane and are here described in more detail. An SLB is formed on a solid support (sensor surface), typically coated with SiO_2, by adsorption and rupture of liposomes leading to the formation of an extended planar bilayer. The rupture of the adsorbed liposomes is for most lipid compositions initiated at a certain surface coverage due to mechanical strain between adjacent liposomes. The initial rupture of liposomes form patches of bilayers which in turn fuse and form a continuous SLB.[11,59,60] SLBs could be formed over large areas (in the order of cm^2) and are fluid in their nature. The simplest SLB consist of only one type of lipids. From this starting point the complexity could be increased by changing the lipid composition or the membrane morphology. The latter typically occur when an amphiphile is added to the membrane.[61] Since the SLB is placed directly on the support, only separated by a thin layer of water molecules,

the incorporation of transmembrane proteins is difficult. These proteins will lose their mobility in the lipid membrane and their function due to the close proximity to the solid support. To solve this problem the SLB could be formed on a polymer cushion or a tether can be placed between the surface and the membrane.[62-64] Since the SLB is confined to a solid support a variety of surface sensitive techniques can be used for the analyses, *e.g.* AFM, QCM-D and reflectometry.[65,66] In Figure 4 a schematic of a SLB on a QCM-D sensor surface is shown.

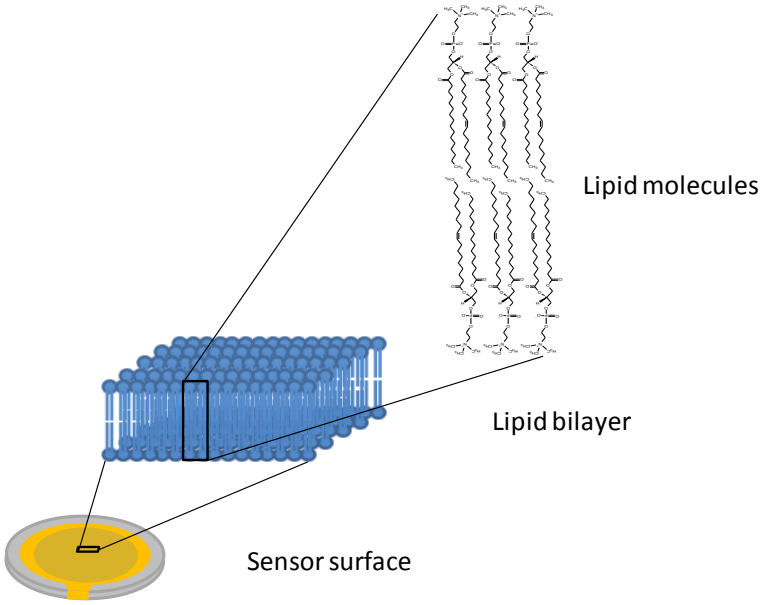

Figure 4. Schematic model of a supported lipid bilayer formed on a QCM-D sensor surface.

3.2.3. Langmuir-Blodgett films

Another commonly used model system of the cell membrane is Langmuir-Blodgett (LB) films[67]. The LB technique, in which the model membrane is formed, can be divided in two main parts. First, an organized layer of amphiphilic molecules (*e.g.* lipids) is formed at an air-water interface (Langmuir monolayer) and second, a substrate is vertically passed through the interface transferring the Langmuir monolayer to the substrate. The substrate can be passed through the monolayer repeatedly, and for each pass an additional monolayer of the amphiphilic molecules is added. In this way it is possible to build multilayered assemblies on the substrate. The Langmuir monolayer, which by itself can be used as model system for the cell surface, is formed inside a through in which the surface area can be altered. This makes it possible to alter the local density and organization of the molecules by regulating the lateral pressure. At a surface pressure of 30 mN/m the lipid packing density is similar to a cell membrane.[68] At this point, drugs or drug loaded NPs can be added to the subphase and the change in surface pressure can be studied.[10] Naturally, Langmuir monolayers cannot be used as a model to study transport processes across a cell membrane since it only consists of one lipid layer and is located at an air-water interface. Despite this, this type of model system can be used to mimic the outer surface of a cell. Other advantages with using lipid monolayers as

a model system, apart from the possibility to control the surface pressure, are that the lipid composition, temperature and subphase content are controlled.[69]

As mentioned, it is possible to form a bilayer on a solid support from the Langmuir monolayer by the LB technique. This is performed by first forming a lipid monolayer on the surface of the subphase and then transferring the substrate first downwards and then upwards through the interphase. The same result can also be obtained by a combination of the LB and the Langmuir-Schaeffer (LS) techniques. In the LS technique[70], a Langmuir monolayer is formed and subsequently a hydrophobic substrate is placed horizontal to the subphase and kept in contact with the Langmuir monolayer for 30-60 s. In this way the Langmuir monolayer is transferred to the hydrophobic substrate. To form a lipid bilayer with a combination of the two techniques, the first layer if transferred by the LB technique and the second by the LS technique.

4. Experimental techniques

In this section the basics of the main techniques used in this work are described. The studied NPs were first characterized by determining their size distribution. This was done with dynamic light scattering, which determines the hydrodynamic diameter of the NPs from their brownian motion in suspension. In the same instrument, also the zeta potential of the NPs was measured through electrophoretic light scattering. QCM-D was used for the main part of the NP-SLB interaction studies. This technique gives the change in hydrated mass when the SLB is exposed to the NPs and information about structural properties of adsorbed material. By combining this technique with reflectometry, which is sensitive to changes in refractive index close to the sensor surface, it was possible to determine the degree of hydration of the adsorbed material. To further understand the interaction processes between the NPs and the model membranes AFM was used to study the surface topography after NP adsorption.

4.1. Light scattering techniques

4.1.1. Dynamic light scattering

Dynamic Light Scattering (DLS), also known as Photon Correlation Spectroscopy (PCS), is a technique that is used to determine the size of colloidal particles or macromolecules in solution.[71] The technique takes advantage of the Brownian motion of particles in solution. Brownian motion is a stochastic process due to collisions with surrounding molecules. When illuminating the suspension with laser light, the light will be scattered by the particles and due to the Brownian motion the intensity of the scattered light will fluctuate over time. The time-dependant fluctuation is measured and used to determine an autocorrelation function. In autocorrelation measurements the signal is constantly compared to itself using a small time shift, τ. The correlation of the signal, G, decays exponentially and the rate is determined by the diffusion of the particles.

$$G = \int_0^\infty I(t)\, I(t+\tau) dt = B + Ae^{-2q^2 D\tau} \qquad (1)$$

In equation 1, B is the baseline, A the amplitude, q the scattering vector and D the translational diffusion coefficient. The scattering vector is calculated according to equation 2 where n is the refractive index of the solvent, λ_0 is the wavelength of the laser in vacuum and θ is the scattering angle.

$$q = \frac{4\pi n}{\lambda_0} sin\left(\frac{\theta}{2}\right) \qquad (2)$$

By measuring the speed of the Brownian motion, the translational diffusion coefficient, D, is obtained (eq 1). Using the Stokes-Einstein equation (eq. 3), this diffusion coefficient can be used to calculate the hydrodynamic diameter, D_H.

$$D_H = \frac{kT}{3\pi\eta D} \qquad (3)$$

In equation 3, k is the Boltzmann constant, T is the temperature and η is the viscosity of the dispersant.

If the sample consists of several size populations it is possible to determine their individual sizes by using distribution algorithms.[72] However, the result depends on several factors such as the relative sizes of the different populations and their relative scattering intensity as well as their polydispersity. The result is given as an intensity distribution where the percentage of the intensity of the scattered light is shown as a function of the particle size. This data could be recalculated to a number distribution. Since the scattering intensity is related to the particle diameter by a factor of 10^6, the presence of large particles will dramatically influence the intensity distribution while small particles will have a much smaller effect.

4.1.1. Electrophoretic light scattering

By electrophoretic light scattering it is possible to determine the zeta potential of nanoparticles. Around a charged particle there exist layers of counter ions, *i.e.* ions of opposite charge. In the inner layer, the ions are tightly associated with the particle (stern layer) and in the outer layer the ions are more diffusely associated. Inside the diffuse layer there is a boundary called the slipping plane. Within the slipping plane the ions move together with the particle as a stable entity in an electric field. The potential at the slipping plane is commonly referred to as the zeta potential or mean surface charge. To determine the zeta potential, an electric field is applied across a suspension of the particles. When equilibrium between the electric and the opposing frictional forces is reached, the particles are travelling at a constant velocity (electrophoretic mobility, U_E). This velocity is measured by laser doppler velocimetry. The electrophoretic mobility could then be used to calculate the zeta potential by using the Henry equation (eq. 4)

$$U_E = \frac{2\varepsilon z f(ka)}{3\eta} \qquad (4)$$

In equation 4, ε is the dielectric constant, z is the zeta potential, *f*(ka) is Henry´s function and η is the viscosity. When measurements are performed in aqueous media at moderate electrolyte concentrations, Henry´s function can be estimated to 1.5 according to the Smoluchowski approximation.

4.2. QCM-D

Quartz crystal microbalance with dissipation monitoring (QCM-D) is a technique that measures small mass changes on a sensor surface, and the viscoelastic properties of the attached material. It is a well established surface sensitive technique which has been used to study the formation of SLBs[59,73] and their biomolecular interactions[74]. The sensitivity of the technique is very high and masses in the order of ng/cm^2 can be detected. The technique is built upon a piezoelectric quartz crystal. This means that when the crystal is subjected to mechanical stress electric charges are generated on its surface, and when an electric field is applied the crystal is strained. In most QCM-D setups, AT-cut quartz crystals are used and the

surfaces of the disc shaped sensors are covered with thin metal electrodes. An AT-cut crystal is cut in an angle of 35.25° from its optical axis and oscillates in thickness shear mode when subjected to an oscillating electric field. Resonance occurs when the frequency of the applied field corresponds to the fundamental frequency of the crystal or to a corresponding overtone.[75] Mass associated to the sensor surface induce a decrease in the resonance frequency. If the mass ($m_{acoustic}$) is small compared to the mass of the crystal, evenly distributed in a thin layer, rigidly coupled and does not slip, it is proportional to the induced shift in frequency (Δf_z). This relationship is described by the Sauerbrey relation (eq. 5).[76]

$$m_{acoustic} = C \cdot \frac{\Delta f_z}{z} \qquad (5)$$

C, the mass sensitivity constant, is -17.7 ng/(cm^2 Hz) for an AT cut crystal with a fundamental frequency of 5 MHz and z (1, 3, 5, 7, 9, 11 or 13) is the number of the harmonic. In non rigid films, shear acoustic waves propagates differently than in the quartz crystal. Due to this, the crystal and the attached film cannot be considered as one unit and the Sauerbrey equation is no longer valid. Apart from the frequency, the dissipation factor (D) is also measured in the QCM-D technique. This factor derives from decay rate of the voltage over the crystal when the driving voltage is turned off and is described by the following relation (eq. 6).[77]

$$D = \frac{E_{dissipated}}{2\pi \cdot E_{stored}} \qquad (6)$$

$E_{dissipated}$ is the energy dissipated during one period of oscillation and E_{stored} is the energy stored in the oscillating system. The dissipation factor correlates to the viscoelastic properties of the attached material, very rigid films have low dissipation and loosely attached materials generates high dissipation. In QCM-D instruments all mass associated with the sensor surface are measured, not only the "dry" mass. This property is evident when studying the formation of SLBs by liposome rupture. First, intact liposomes adsorb to the sensor surface generating large shifts in both frequency and dissipation. The large responses are due to the floppy structure on the surface and the large amount of liquid associated with the intact liposomes, both in their interior and between adjacent liposomes. Second, the liposomes start to rupture and release the enclosed liquid to the surroundings, a process that lead to a decrease in the frequency and dissipation shifts. Finally, when the SLB have been formed, the frequency and dissipation shifts reach characteristic values of -26 Hz and < 0.5 respectively.

4.3. Reflectometry

In contrast to QCM-D, reflectometry is an optical technique. This difference in sensing principle makes the two techniques an excellent complement to one and other. While QCM-D senses the hydrated mass ($m_{acoustic}$) associated to the sensor surface, optical techniques only detects the "dry" mass (m_{optic}). By combining the two techniques the degree of hydration of the adsorbed material could be determined.[66] Reflectometry is based on the fact that the optical properties, the reflectivity, of a surface changes when mass is adsorbed to it. The surface under study is illuminated through a prism with monochromatic, plane polarized light

at a certain angle of incidence. The reflected light is split in two components with different polarizations and the intensity ratio (S) of these two polarizations is monitored. This ratio changes when adsorption/desorption processes occurs at the surface and the relative changes is given as the output ΔR.

$$\Delta R = \frac{S - S_0}{S_0} \quad (7)$$

S_0 corresponds to the initial intensity ratio, *i.e.* only buffer. The optical output, ΔR, is related to the adsorbed mass through the following equations

$$\Delta R = d \cdot (n - n_0) \cdot A \quad (8)$$

$$m_{optic} = \frac{d \cdot (n - n_0)}{dn/dc} \quad (9)$$

Where d is the thickness of the adsorbed layer and n its refractive index, A is the sensitivity factor and dn/dc id the refractive index increment of the adsorbed material.[78] In this work a prototype instrument of combined QCM-D and reflectometry has been used. This enables the two techniques to be used simultaneously, at the same sensor surface, and the degree of hydration (H) to be determined.

$$H = \frac{m_{acoustic} - m_{optic}}{m_{acoustic}} \quad (10)$$

4.4. AFM

Atomic force microscopy (AFM) belongs to a family of scanning probe microscopy techniques which stems from the scanning tunneling microscopy (STM) technique developed in the early 1980´s. The invention of the AFM in 1986 made it possible to analyze all surfaces, not only the ones that are electrically conductive as is the case for STM. This advantage, together with the possibility to perform analyses in liquid environments, opened up the door to the field of biology. From now on it was possible to resolve features on biological samples much smaller than the optical diffraction limit.[79]

The three main components in a typical instrument are the cantilever to which a sharp tip is attached, an optical system used to detect cantilever deflection, the piezoelectric translation system and feedback circuitry (Figure 5). The cantilever is usually fabricated from silicon or silicon nitride and has a spring constant between 0.01 and 100 N/m. The piezoelectric translation system typically consists of a tube made from a piezoelectric ceramic. This system raster scans the tip over the sample, where the movements can be controlled with high accuracy in three dimensions. The optical deflection system measures the bending of the cantilever which in turn is dependent on the tip-sample interaction (*i.e.* “force”).

An AFM analysis can be performed either in contact or in tapping mode. In contact mode the tip will be in constant contact with the surface, and the image is created from either the bending of the cantilever or the movements of the cantilever base in the z-direction. These two ways of detection are called constant height and constant force, respectively. In the latter

case the data from the optical deflection system is fed into a feedback loop, which keeps the deflection of the cantilever (*i.e.* the force) constant. In tapping mode the cantilever is made to oscillate close to its natural resonance frequency. When the tip comes in close contact with the surface, the oscillation amplitude will change due to tip-sample interaction. When the tip is scanned across a surface, the amplitude is kept constant by the feedback loop, and the necessary scanner height adjustments are used to form an image. In this work, only contact mode AFM at constant force has been applied.

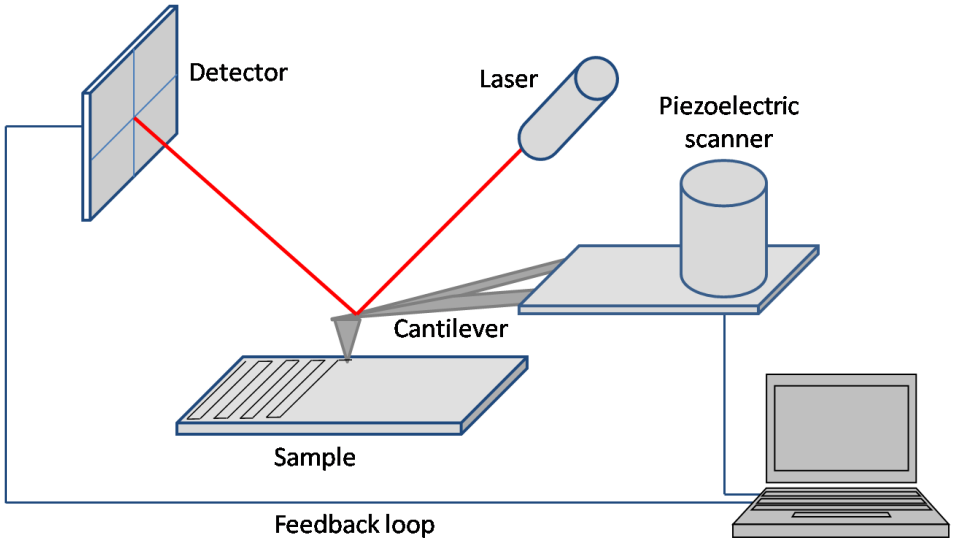

Figure 5. Schematic illustration of an AFM setup.

The AFM can also be used for force spectroscopy, *i.e.* the force is measured versus the distance between the tip and the sample at a fixed lateral position. With force spectroscopy, intermolecular forces can be measured and the force spectra can indicate the length or thickness of an investigated object.[80] This is a good way to detect the presence of a SLB on a surface. Since the membrane is very flat and faithfully follows the contours of the underlying substrate, a topographic image will not easily reveal its presence. The outcome of the force spectra will however be different if a membrane is present compared to the bare surface.

5. Results

This chapter summarizes the results presented in the appended papers. In brief, different insulin-loaded NPs based on polyelectrolytes, *i.e.* PECs, were first characterized with respect to size and charge and then with respect to their lipid membrane interactions using the techniques described in the previous chapter.

In Paper I, differently charged SLBs were exposed to one type of PEC and the NP-membrane interactions were analyzed with QCM-D and a combined QCM-D/reflectometry setup. In addition, optical modeling of the sensitivity factor was performed. It was found that the positively charged PECs selectively adsorbed to negatively charged membranes and that the degree of negative charge determined their structural deformation upon adsorption.

In Paper II, the responsiveness of several different PECs towards a reducing agent and a decrease in pH were evaluated by QCM-D and AFM. The results showed that the PECs collapsed upon adsorption without disrupting the underlying membrane and that PECs containing disulfide linkages in the polymer backbone dissociated upon addition of a reducing agent. A similar response was obtained when the ambient pH was decreased.

In Paper III an attempt to make a PEC more repellant, by introducing a PEG-containing polymer, was evaluated. It was clear that the PEG-ylation needed to be altered since the desired effect was not obtained.

5.1. Characterization of nanoparticles

As a first step, all types of NPs that have been analyzed in this study were characterized with respect to their size and charge. These types of analysis were mainly performed using dynamic (size) and electrophoretic (charge) light scattering. The preferred sample should have a low polydispersity and a low variation of the mean size between different batches. In Paper I, the investigated NP (referred to as NP-HI) was analyzed with both DLS and SEM (Figure 6). The hydrodynamic diameter was determined to approximately 220 nm by DLS. In contrast, the size revealed by electron microscopy was much smaller ($d < 100$ nm). The large difference between these results was due to the hydration of the analyzed NPs. Prior to the SEM analysis, the sample are dried and the analysis is preformed under vacuum conditions whereas the DLS analysis is preformed in liquid. Naturally, these different sample preparations yield particles with different size.

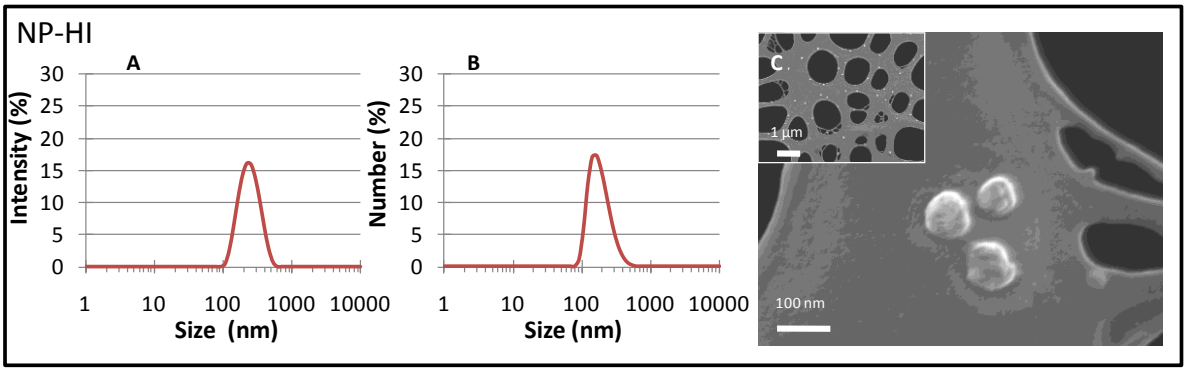

Figure 6. Characterization of NP-HI with respect to size. (A) Intensity and (B) number distributions obtained by DLS. (C) SEM images of different magnifications (20 000x (inset) and 400 000x) visualizing the studied NPs. (Paper 1)

Apart from the presented example, all size characterizations of NPs were solely based on DLS analyses. This method was selected because of the possibility to perform the analyses under the same conditions as for the following QCM-D, relectometry, and AFM experiments as well as of the high throughput.

5.2. Nanoparticle interaction with model membranes

After the nanomaterials were characterized by light scattering techniques and it was concluded that the materials were well defined, their interaction with model lipid membranes were analyzed.

Three model membranes, based on differently charged phospholipids, were used. SLBs were formed on a SiO_2 support using positively charged (ζ-potential: 22 ± 0.8 mV) POPC:POEPC (3:1), neutral/slightly negatively charged (ζ-potential: -0.3 ± 1.0 mV) POPC, and negatively charged (ζ-potential: -26 ± 1.2 mV) POPC:POPS (3:1) liposomes. The formed SLBs were assumed to have similar charge as the corresponding liposomes and they typically yielded QCM-D responses which are characteristic for high quality SLBs; $\Delta f = -26$ Hz and $\Delta D < 0.5$.

In a next step, NPs were added to the formed SLBs and the outcome of the NP-SLB interaction was studied in real-time with QCM-D. A schematic of the experimental setup is presented in Figure 7. QCM-D was chosen to be the first method of analysis due to the gained structural information of the NP-SLB interactions and the relatively high throughput. From the recorded data, specific experiments were selected to be performed with other surface based techniques, *e.g.* AFM or combined QCM-D/reflectometry.

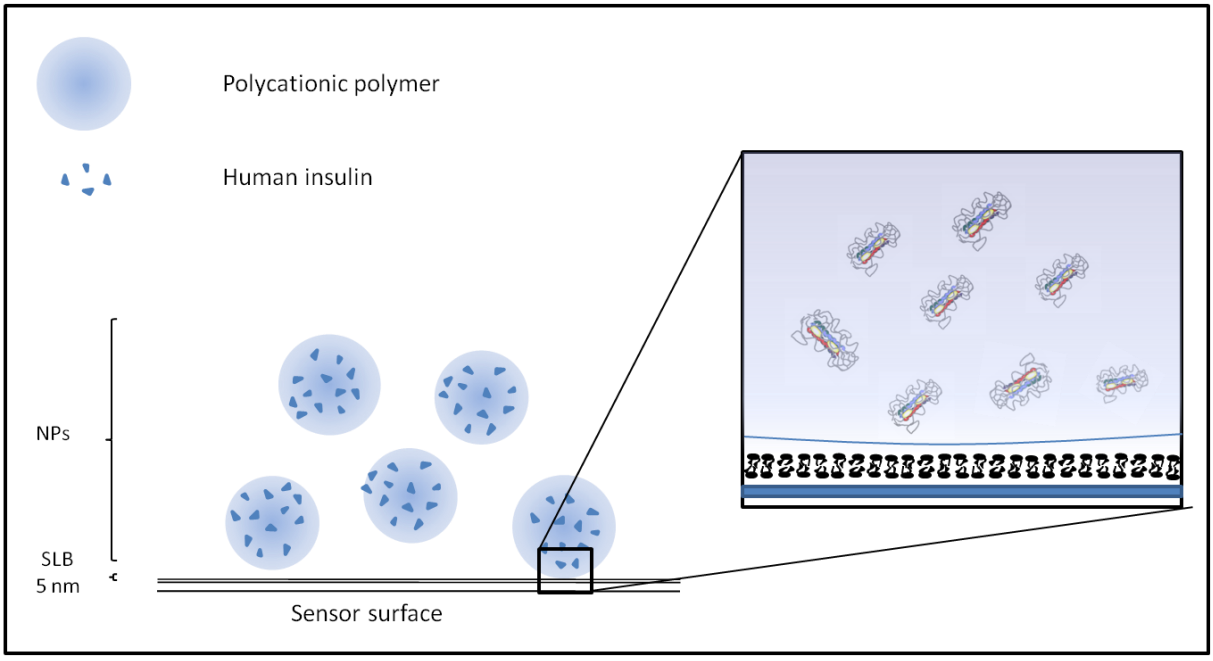

Figure 7. Schematic of the experimental setup where a formed SLB on a sensor surface is exposed to insulin-loaded PECs.

In paper I, the studied NP (referred to as NP-HI) selectively interacted with the negatively charged POPC and POPC:POPS membranes. This was an expected result due to the positive charge of the NP-HI (ζ-potential: 26 ± 2 mV). However, the QCM-D analysis showed that the adsorbed NP-HI had different structural confirmations depending on the degree of negative charge of the membrane.

QCM-D responses obtained when NP-HI adsorbed on a negatively charged POPC:POPS (3:1) membrane suggested the formation of a thin and fairly rigid structure on the membrane. A more loose structure was formed on a plain POPC membrane. This layer was characterized by high ΔD values and by large spreading between different harmonics compared to the POPC:POPS (3:1) membrane. To further investigate the structural differences between these two cases, they were analyzed in a combined QCM-D/reflectometry setup (Figure 8). The aim of this analysis was to compare the two different structural arrangements of the adsorbed NPs with respect to their degree of hydration. For NP-HI layers adsorbed on a POPC:POPS (3:1) membrane, $m_{acoustic}$ and m_{optic} were calculated as described in section 4.2 and 4.3 respectively, and the degree of hydration was determined to approximately 70%. The thicker layer formed by adsorption of NP-HI to a plain POPC membrane generated a decrease in the optical signal (ΔR). This result suggests, in contrast to the QCM-D data that mass is lost from the surface. Hence m_{optic} of the adsorbed NP-HI could not be determined for this case. The reason behind the negative optical signal was elucidated through optical modeling of the system and was found to be related to the thickness of the adsorbed material and not mass loss. The optical model and the modeled data are described in more detail in section 5.4.

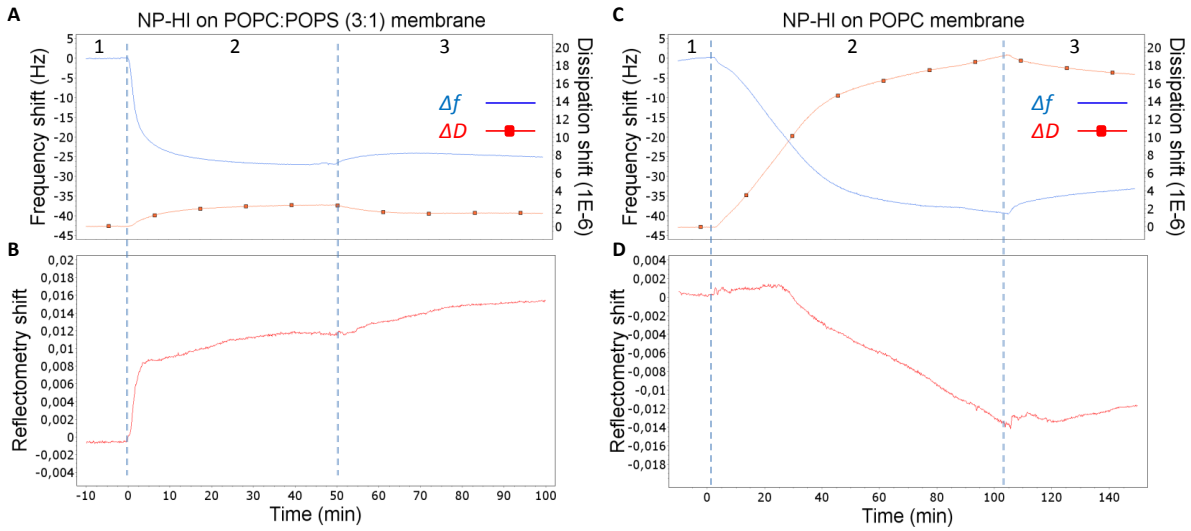

Figure 8. QCM-D (z = 9) and corresponding reflectometry data of the adsorption of NP-HI to a POPC:POPS (3:1) membrane (A, B) and a plain POPC membrane (C, D). The plots show (1) a baseline after membrane formation, (2) adsorption of NPs and (3) buffer rinse. (Paper I)

In both paper II and III, PEG-ylated NPs were compared with non PEG-ylated NPs. In one case (Paper II) the PEG-ylation reduced the positive surface charge of the NPs and to a certain extent prevented adsorption to a POPC:POPS (3:1) membrane. This result was in agreement with the general idea that PEG-ylation makes NPs more repellant and therefore prolongs the circulation half-life of the NPs. It was also evident that the fraction of PEG-ylated NPs that adsorbed to the membrane generated a more viscous arrangement on the surface compared to the non PEG-ylated. This was most likely due to the PEG-chains that extend from the polymer backbone. In contrast to the results presented in Paper II, the comparison of PEG-ylated and non PEG-ylated NPs in Paper III revealed that the desired effect was not obtained. In this study, where the NPs are referred to as NP-HI (same NP as in paper I) and PEG-NP-HI, the change in zeta-potential was small and both types of NPs adsorbed to the same extent to a POPC:POPS (3:1) model membrane. No adsorption occurred on a POPC:POEPC (3:1) membrane. The QCM-D data are shown in Figure 9. From the results it was possible to conclude that the PEG-ylation was not successful and the approach needs to be altered to obtain the desired effect.

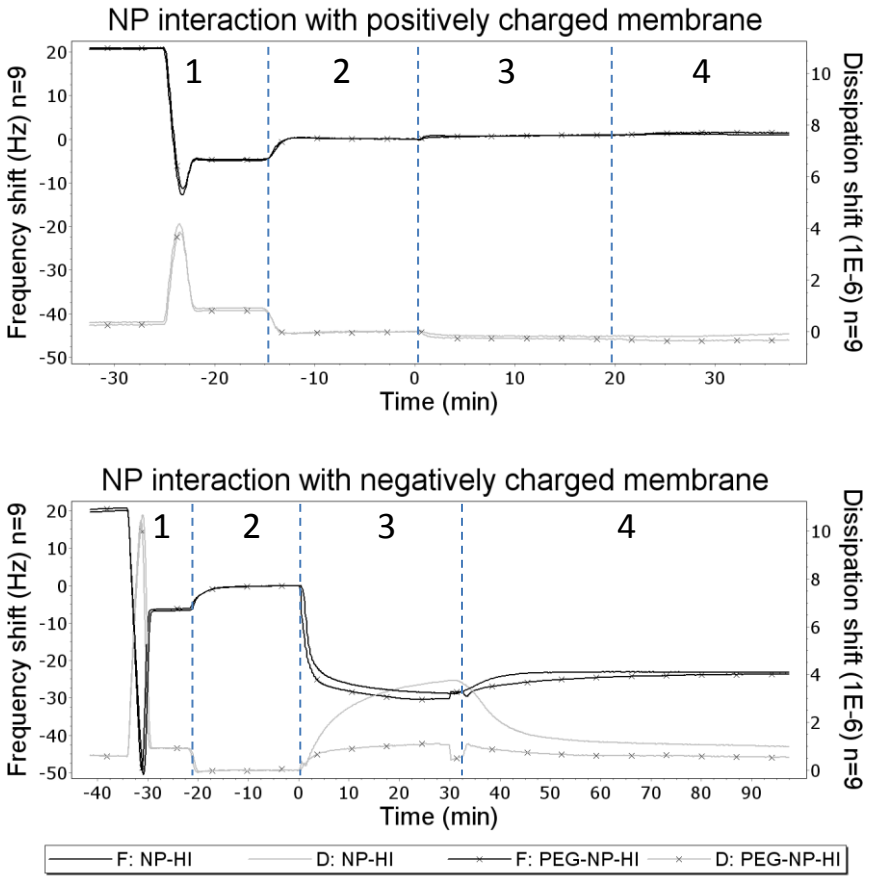

Figure 9. QCM-D data of adsorption of NP-HI and PEG-NP-HI on a positively charged POPC:POEPC (3:1) membrane and a negatively charged POPC:POPS (3:1) membrane. Both plots show the following sequence of events: (1) SLB formation, (2) buffer exchange, (3) addition of NPs and (4) buffer rinse. (Paper III)

5.3. Bioreduction of nanoparticles by mimicking intracellular degradation

When NPs are taken up by a cell through endocytosis they are subjected to a low pH in the late endosome. This change in the surrounding environment can be utilized to trigger the release of the drug from its carrier. Similarly, the intracellular reductive environment can be used to disintegrate the carrier to promote drug release. In paper II, three different responsive PECs based on poly(amido amine)s were produced and evaluated. These NPs were responsive to reducing agents due to the presence of disulfide linkages[81] in the backbone of the polymers. The NPs, which are referred to as NP 1-3, were also designed to disintegrate when pH was decreased from physiological to about 5 due to a strong decrease in the charge attraction between the polymer and the protein at this low pH. A fourth NP (NP 4), without disulfide linkages in the polymer backbone, was included in the study as a control. In this study the NPs were first adsorbed to a preformed POPC:POPS (3:1) membrane. Subsequently, the ambient conditions were altered either by adding a reducing agent (glutathione) or by decreasing the pH.

As expected, NPs containing disulfide linkages in the polymer backbone responded to the presence of glutathione while the NP without disulfide linkages was unaffected by the addition of glutathione. The previously adsorbed NPs containing disulfide linkages dissociated from the membrane. The QCM-D frequency shift Δf suggested that the intact lipid membrane remained on the surface. The percent of the adsorbed NP mass that dissociated from the surface in the four different cases are presented in Figure 10A.

NP 1, was evaluated with respect to its pH-sensitivity. After adsorption to a POPC:POPS membrane and subsequent buffer rinse, the pH was decreased from 7.3 to 5.1 using a pH-gradient lasting for one hour. The result shows that mass start to dissociate from the surface at a pH of approximately 6.5. After this point, a rapid mass release occurs until a pH-value of about 6. Finally, at pH 5.1, the mass loss has leveled out at a level where 20 % of the initial amount of mass is left on the membrane. The result is presented in Figure 10B.

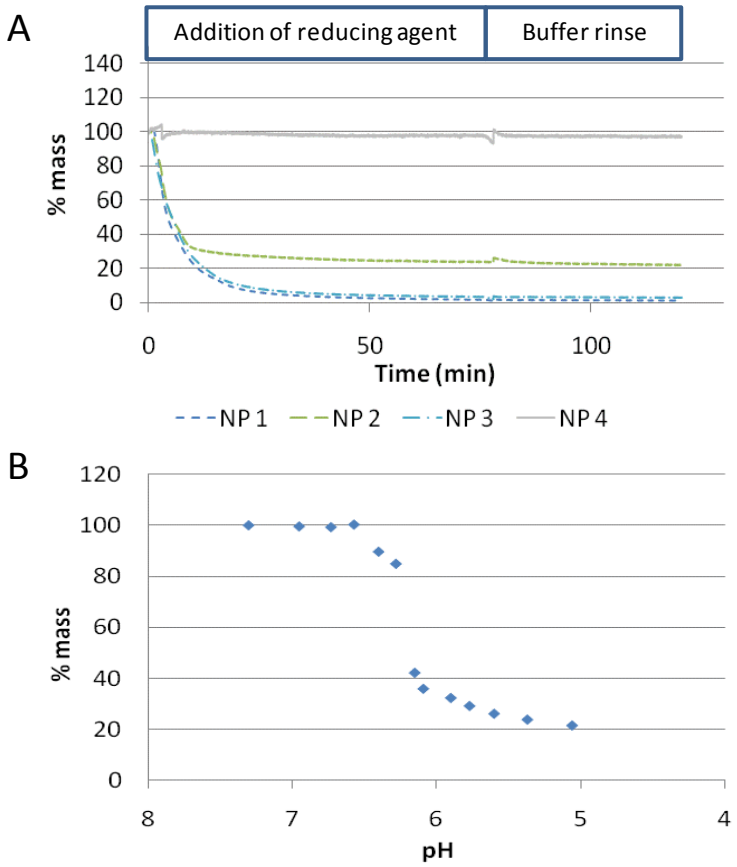

Figure 10. Response of the adsorbed NPs to (A) addition of a reducing agent (glutathione) and (B) a decrease in pH. (Paper II)

The scenario suggested by the interpretation of the QCM-D data was further strengthened by AFM measurements. The adsorption of NP 1 to the model membrane and its response to glutathione was evaluated by imaging after the formation of the SLB, after adsorption of NPs, and after addition of glutathione (Figure 11). Corresponding force spectra were also recorded. The bare SLB was detected by a kink in the force spectrum originating from when the tip was pressed through the SLB during its approach towards the surface. After adsorption of NP 1, this characteristic kink corresponding to the SLB was still present. In addition, forces were

exerted on the tip several tens of nanometers away from the surface. This event in the force spectrum reveals the presence of the adsorbed NP material. Another main difference in the force spectrum after addition of NPs was the pull off force. Before addition of NPs the pull off was a distinct event where the tip snapped off the surface, while after addition of NPs the pull off occurred much more slowly. After addition of glutathione two different regions were revealed on the surface. Although the SLB could be detected in both, the force spectra suggested that in one of the regions an additional thin layer of NP material was present.

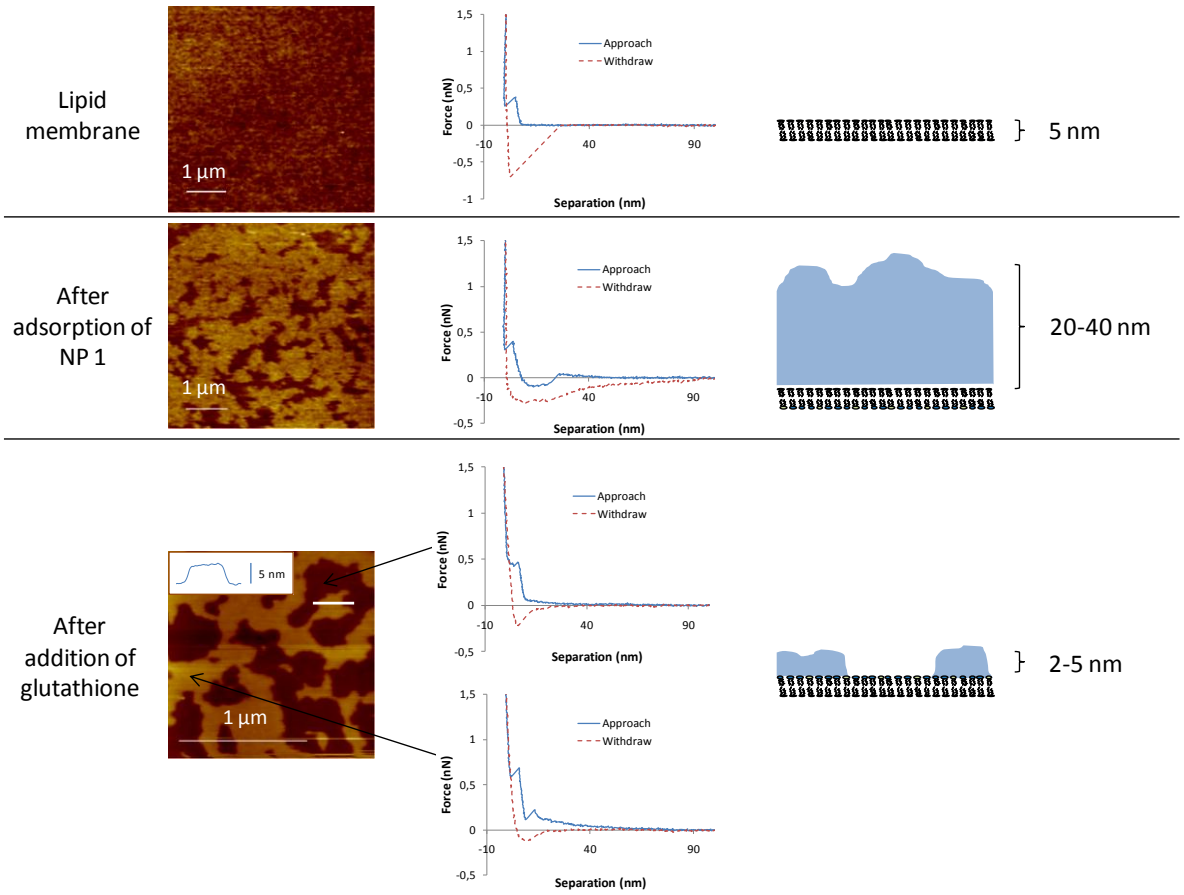

Figure 11. AFM images, corresponding force spectra and schematic models (not to scale) of the bare lipid membrane (z-range 1.6 nm), after adsorption of NP 1 (z-range 3.0 nm) and after addition of glutathione (z-range 10 nm). The cross section, shown in the inset, corresponds to the white bar in the image. (Paper II)

By combining the data obtained from QCM-D and AFM after NP adsorption and after subsequent addition of glutathione, a schematic model of the surface was made. The model, which is shown to the right in Figure 11, shows that the NPs collapse into a layer much thinner than the hydrodynamic diameter of the NPs (d = 165 ± 5 nm) when adsorbed to a POPC:POPS membrane. After addition of glutathione only a few nanometers thick layer which partially covered the surface remained.

Both the presented cases where adsorbed NPs dissociate from the surface due to particle disintegration were most likely associated with release of the insulin drug load. However, to follow the release of insulin from its carrier other methods must be applied which in most cases require labeling of the insulin molecules.

5.4. Modeling the sensitivity factor

In paper I, a prototype instrument which combines QCM-D and reflectometry on the same sensor surface was used. Due to the complementary sensing principles of the two techniques it was possible to determine the degree of hydration of the adsorbed material. When the investigated NP-HI was adsorbed to a negatively charged POPC:POPS (3:1) membrane, the analysis was successful. However, when the same experiment was repeated on the less negatively charged POPC membrane an unexpected result was obtained. The reflectometry signal (ΔR) decreased when the NP-HI was introduced in the system (see Figure 8D, section 5.2). The first, and most straightforward, interpretation of this result was that material was lost from the surface. This data was contradicted by the result of the simultaneous QCM-D analysis, which showed mass adsorption (negative Δf) and a viscous structure (high ΔD). It was elucidated that the decrease in ΔR upon adsorption of NPs was due to the thickness of the adsorbed layer. A thick adlayer gives rise to a negative sensitivity factor (A) and hence ΔR was negative. The dependence of the sensitivity factor on the thickness was studied by optical modeling using the software Wvase32 (J.A. Woollam Co. Inc., USA). First a model of the experimental system was created. The different layers included in the model were the underlying Ti (bulk, partially oxidized), 110 nm of SiO_2 (thickness determined by ellipsometry), the SLB, the adsorbed NP-HI and the ambient medium (Figure 12A). After the model was created the sensitivity factor was modeled for different thicknesses of the NP-HI layer (Figure 12B). The obtained result showed that the sensitivity factor decreased rapidly with increasing thickness of the adsorbed layer and was negative between 100 and 400 nm.

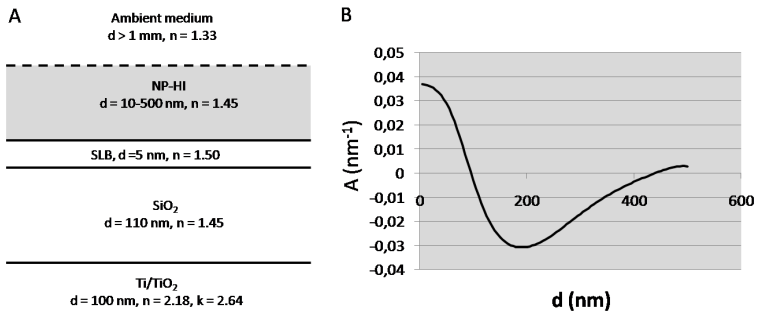

Figure 12. Modeling of the reflectometry sensitivity factor A. (A) The different layers included in the model and their respective thicknesses (d) and optical properties (n, k). (B) Plot showing how the calculated sensitivity factor varies as a function of the thickness (10-500 nm) of the NP-HI layer (grey in (A)). (Paper I)

Modeling of the QCM-D data with Q-tools (Q-sense, Sweden) showed thicknesses of about 100 nm in the experiments where ΔR decreased upon NP-HI adsorption. Hence, the observed decrease in the optical signal most likely is due to a too thick adlayer on the surface. This significant dependence of the sensitivity factor on the thickness of the adsorbed material limits the use of the reflectometry analysis since it is only accurate for very thin layers.

6. Concluding remarks and outlook

Nanomedicine is an emerging field of research and the expanding development of nano-sized drug carriers motivates the establishment of an early phase *in vitro* screening platform. The methodology presented in this thesis, where surface sensitive techniques are used to investigate the interaction between SLBs and NPs, is suggested as an in vitro screening tool to evaluate and further characterize NPs, *e.g.* with respect to different surface chemistries and functionalities *e.g.* drug release mechanisms.

In this work, several different NPs have been investigated and evaluated with respect to their intrinsic properties. For example, it has been concluded that PECs undergo structural rearrangements upon adsorption to an oppositely charged membrane and that the degree of rearrangement relates to the membrane charge. Hence, the electrostatic attraction between the NP and the model membrane is an important parameter in the NP-SLB interaction process. The chemical composition of NPs has also been addressed, *e.g.* the effect of introducing PEG in the NP formulation has been evaluated. In the two examples that were presented, one approach to PEG-ylate NPs clearly increased the stealth properties of the formulation while no significant effect was seen in the other. Furthermore, the responsiveness of NP formulations towards a reducing environment and a decrease in pH has been evaluated after adsorption to a model membrane. These analyses showed a clear NP response when adding a reducing agent or when decreasing the ambient pH. In both cases NPs dissociated from the surface due to destabilization of the formulation. Taken together, new information about the investigated NPs (PECs) has been gained and the use of the experimental platform has been shown. The experimental platform has a clear potential for further development and could possibly be an important substitute for early cell culture assays and eventually also contribute to decrease the number of *in vivo* experiments.

The model membranes used in this work were supported lipid membranes consisting of one or two types of lipids. Although native cell membranes are much more complex, with additional constituents such as membrane proteins and polysaccharides, these model membranes represent the basic structure of the cell membrane, the lipid bilayer. One natural development of this experimental platform is to use more complex model membranes to further mimic the native cell membrane. In this way there would be a smaller gap between these studies and corresponding cell culture assays. The main step to overcome is to be able to form supported membranes using vesicles containing other molecules apart from lipids. There is, however, the possibility to incorporate the additional membrane constituents after formation of the supported lipid bilayer. One specific application of interest is to investigate the targeting properties of drug carriers to specific membrane receptors present in the model membranes. Another approach to create a more realistic model system would be to prepare liposomes from cultured cells and immobilize these on a surface. In this way, model systems for specific cell types could be used depending on the purpose of the studied NPs. The following cell types would be of special interest: (1) Epithelial cells present in *e.g.* lung, nose, or small intestine. When delivering drugs through the pulmonary, nasal, or oral route it must pass through

epithelial cells to reach the bloodstream. (2) Micro- and macrovascular endothelial cells present in micro capillary and larger blood vessels respectively. The NPs would encounter these cell types after injection in the bloodstream or after passage through an epithelial cell lining. (3) Brain microvascular endothelial cells which form a very tight barrier (the blood-brain barrier) separating the brain from the rest of the body. If drugs are to be delivered to the brain, the blood-brain barrier needs to be overcome. Preferably the outcome of the surface based analysis of the interaction between the nanodrugs and the native liposomes should be correlated with a response obtained when the nanodrugs are exposed to the corresponding living cell cultures (*e.g.* uptake of NPs or cell count). If such a correlation is found, the screening of novel drug carriers could be made much more efficient.

Furthermore, by applying additional surface sensitive techniques, it would be possible to learn even more about the fate of the NPs when adsorbed to the model membrane. Potentially, the drug release from the NPs once adsorbed to the model membrane could be evaluated. One preferred method to use is total internal reflection fluorescence (TIRF) microscopy. This technique gives a very low background signal since the fluorophores are only excited close to the surface because of the exponential decay of the evanescent field, compared to ordinary fluorescence microscopy. The main drawback is that the technique requires labeling of the drug molecules.

In this work only NPs developed for drug delivery purposes have been evaluated. However, the described experimental platform is also promising for studies other types of nanomaterials. For example, the methodology could be used to relate NP characteristics to membrane disruption. Hence, the toxicity of NPs could be assessed.

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